Application
for
United States Letters Patent

To all whom it may concern:

Be it known that Saul J. Silverstein, Paula W. Annunziato, Anne A. Gershon, Octavian Lungu

have invented certain new and useful improvements in

VZV ORF29p PROTEIN-RELATED COMPOSITIONS AND METHODS

of which the following is a full, clear and exact description.

VZV ORF29P PROTEIN-RELATED COMPOSITIONS AND METHODS

This application claims the benefit of copending U.S. Provisional Application No. 60/177,901, filed January 25, 2000, the contents of which are hereby incorporated by reference.

The invention described herein was made with Government support under grant numbers AI-01409 and AI-124021 from the National Institutes of Health. Accordingly, the United States Government has certain rights in this invention.

Background of the Invention

Throughout this application, various publications are referenced by arabic numbers within parentheses. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Varicella-Zoster Virus ("VZV") infects dorsal root ganglia ("DRG"), enters latency, and may later reactivate to cause zoster. Studies have detected VZV in specific sites at different stages of infection. VZV DNA is present in the oropharynx (27) and in peripheral blood mononuclear cells ("PBMCs") of patients with chickenpox (3, 16, 20). Virus DNA, the glycoproteins gE and gB, and the immediate-early protein 63 ("IE63p") are found in skin biopsy samples obtained from patients with chickenpox or zoster (1, 23-25). VZV is found in keratinocytes, antigen-presenting

cells, and endothelial cells during acute zoster (23, 25) and in keratinocytes and inflammatory cells during chickenpox (1). VZV is present in neurons and satellite cells of DRG years following primary infection (6-8, 12, 17, 22) and has been observed by electron microscopy in sensory nerves during zoster (10). Other details of VZV pathogenesis remain speculative, including how the virus spreads from respiratory epithelial cells to PBMCs, keratinocytes, and DRG. Because PBMCs, sensory nerves, and epithelial cells are in close proximity in the dermis and epidermis, the skin is likely the site where this virus enters the nervous system.

By analogy with herpes simplex virus ("HSV"), it is thought that VZV transcription is temporally regulated. Immediate-early ("IE") genes are expressed first, followed by early ("E") genes and lastly late ("L") genes (5). Some VZV proteins encoded by IE and L genes are incorporated in the virion, including trans-activators such as IE63p and structural proteins such as gC (14, 15). ORF29p (for open reading frame 29 protein), the major VZV DNA-binding protein, is encoded by a putative E gene and is not detected in purified virions (13). ORF29p is also referred to herein as "29p protein", "ORF29p protein", and "VZV ORF29p protein." During latency, VZV exhibits limited gene expression (6-9, 22), with the accumulation of specific IE and E gene-encoded proteins in neurons (18, 19). During reactivation, all kinetic classes of VZV genes are expressed in neurons (18). Whether VZV is in the lytic or latent state is reflected by the localization of expressed VZV gene products. VZV IE and E proteins that are present in both the nucleus and cytoplasm during productive infection are detected only

in the cytoplasm of neurons during latency (18).

Early observations suggested that there were inclusion bodies in endothelial cells present in varicella lesions (29). However, there was no known association between VZV histology and viral etiology at that time.

Summary of the Invention

The present invention provides a first composition of matter comprising 29p protein having bound thereto an agent whose delivery into a eukaryotic cell is desired, which composition of matter enters the cell upon contact therewith.

The present invention also provides a second composition of matter comprising a 29p protein having operably affixed thereto a lipid-soluble moiety which permits the protein to be anchored to a lipid membrane.

The present invention also provides a lipid vesicle comprising the second composition of matter anchored thereto via its lipid-soluble moiety, such that the 29p protein is situated on the vesicle's outer surface and facilitates delivery of the vesicle's contents into a eukaryotic cell when the vesicle is contacted therewith. The present invention further provides a monoclonal antibody which specifically binds to 29p protein.

The present invention further provides a method for delivering an agent into a eukaryotic cell comprising contacting the agent with the cell, wherein the agent has bound thereto 29p protein which enters the cell upon contact therewith, thereby delivering the agent into the cell.

The present invention further provides a method for causing a eukaryotic cell to secrete a desired protein in the form of a fusion protein, comprising introducing into the cell a vector for expressing a fusion protein that

comprises the desired protein and 29p protein operably affixed thereto, whereby the cell expresses the fusion protein and the 29p protein thereof permits the fusion protein's exit from the cell, thereby causing the cell to secrete the desired protein in the form of a fusion protein.

The present invention further provides a pharmaceutical composition comprising (a) a composition of matter comprising 29p protein having bound thereto a therapeutic or prophylactic agent, which composition of matter enters a eukaryotic cell upon contact therewith, and (b) a pharmaceutically acceptable carrier.

The present invention further provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier, and a lipid vesicle comprising (a) a therapeutic or prophylactic agent therein, and (b) a 29p protein having operably affixed thereto a lipid-soluble moiety, which protein (i) is anchored to the vesicle via its lipid-soluble moiety, (ii) is situated on the vesicle's outer surface, and (iii) facilitates delivery of the agent into a eukaryotic cell when the vesicle is contacted therewith.

The present invention further provides a method for treating a subject afflicted with a disorder comprising administering to the subject a therapeutically effective amount of the first or second pharmaceutical composition, wherein the therapeutic agent therein is known to ameliorate the disorder.

The present invention further provides a method for

inhibiting the onset of a disorder in a subject comprising administering to the subject a prophylactically effective amount of the first or second pharmaceutical composition, wherein the prophylactic agent therein is known to inhibit the disorder's onset.

The present invention further provides a nucleic acid molecule which hybridizes to at least a portion of a nucleic acid molecule encoding 29p protein.

The present invention further provides a method for detecting the presence of a 29p protein-encoding nucleic acid molecule in a sample comprising the steps of (a) contacting the sample with the instant detectable nucleic acid molecule under conditions permitting it to hybridize to a 29p protein-encoding nucleic acid molecule if present in the sample, and (b) detecting the presence of any detectable nucleic acid molecule so hybridized, thereby detecting the presence of a 29p protein-encoding nucleic acid molecule in the sample.

Finally, the present invention provides a method for quantitatively determining the amount of 29p protein-encoding nucleic acid molecule in a sample comprising the steps of (a) contacting the sample with the instant detectable nucleic acid molecule under conditions permitting it to hybridize to any 29p protein-encoding nucleic acid molecule present in the sample, (b) quantitatively determining the amount of detectable nucleic acid molecule so hybridized, and (c) comparing this amount to a known standard, thereby quantitatively determining the amount of 29p protein in the sample.

Brief Description of the Figures

FIG. 1. Immunohistochemical detection of ORF29p in skin biopsy samples. Chickenpox (A), zoster (B), and Grover's disease (C) skin lesions were analyzed for ORF29p as previously described (18), with the following exceptions. All washes were performed in Tris-buffered saline, and the signal was developed for 10 min in AP substrate (Vector Laboratories, Inc., Burlingame, Calif.), according to the manufacturer's recommendations, in the presence of levamisole to inhibit endogenous alkaline phosphatase activity. Arrows indicate positive epithelial cells. Magnification, x100.

FIG. 2. Immunohistochemical detection of ORF29p and CD43 in skin biopsy samples. Skin biopsy samples from a patient with chickenpox (A and B) or a patient with zoster (C and D) were probed for the presence of ORF29p (A and C) or ORF29p and CD43 (B and D) as described in the legend to Fig. 1. Gray arrows indicate endothelial cells containing ORF29p. Black arrows indicate cells expressing CD43 that contain ORF29p. Magnification, x600.

FIG. 3. Immunohistochemical detection of ORF29p and gC in skin biopsy samples. Sections of nerves in the dermis underlying chickenpox (A and B) or zoster (C and D) lesions underwent immunohistochemistry for ORF29p (A and C) or gC (B and D) as described in the legend to Fig. 1. Magnification, x400.

FIG. 4. Western blot analyses of VZV and HSV-1 proteins. ORF29p, ORF21p, and ICP8 were detected in mock-infected cell extracts (M Cell) and supernatants (M Sup) or cell

extracts (I Cell) and supernatants (I Sup) infected with the viruses denoted on the left. The proteins were immunoprecipitated and detected using the antibodies denoted on the right. Arrowheads denote the proteins of interest.

FIG. 5. Immunohistochemical detection of ORF29p in hNTs. hNTs treated with VZV-infected cell supernatants and LysoTracker were analyzed by immunohistochemistry for the presence of ORF29p. Gray arrows indicate ORF29p (A), LysoTracker (B), and colocalization of ORF29p and LysoTracker in the merged image (C). White arrows indicate an endocytic vesicle that does not contain ORF29p (B and C). ORF29p is restricted to cyptoplasmic vesicles in the treated hNTs (D). Untreated hNTs do not contain ORF29p (E).

FIG. 6. Protein and nucleotide sequences. ORF29p amino acid sequence and nucleotide sequence encoding it.

Detailed Description of the Invention

This invention is based on the surprising discovery that the Varicella-Zoster Virus protein ORF29p can readily enter and exit eukaryotic cells. This unusual property renders it advantageous as, among other things, a vehicle for delivering agents to, and secreting them from, eukaryotic cells.

Specifically, the present invention provides a first composition of matter comprising 29p protein having bound thereto an agent whose delivery into a eukaryotic cell is desired, which composition of matter enters the cell upon contact therewith.

As used herein, the term "29p protein" shall mean the protein having the sequence identified in Figure 6 or a naturally-occurring variant thereof. As stated above, "29p protein" is alternatively referred to herein as "ORF29p", "ORF29p protein", and "VZV ORF29p protein."

The agent of the first composition can be of any physical category. In one embodiment, the agent is a protein or a peptide. In another embodiment, the agent is a nucleic acid molecule. In a further embodiment, the agent is an organic compound.

An agent that is a "protein" is a polypeptide sequence greater than 10 amino acids in length. An agent that is a "peptide" is a polypeptide having a sequence less than or equal to 10 amino acids in length. Examples of protein agents include, for example, insulin, factors VIII and IX, proteases, alpha-glucosidase,

glucocerebrosidase, adenosine deaminase, and DNAase.

An agent that is a "nucleic acid molecule" can be any nucleic acid molecule, including, without limitation, DNA (e.g., cDNA and genomic DNA), RNA (e.g., mRNA and rRNA), and hybrids thereof. The nucleic acid bases that form nucleic acid molecules can be the bases A, C, G, T, and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA). "Nucleic acid molecules" further include, without limitation, antisense, expression vectors, and catalytic nucleic acids such as ribozymes and DNAzymes.

Organic compounds include, without limitation, nutrients such as vitamins, and organic pharmaceuticals such as analgesics, anesthetics, anticonvulsants, antidiabetic agents, anti-infective agents, antineoplastics, gastrointestinal agents, immunosuppressives, parasympatholytics, and parasympathomimetics. Other organic compounds are well known in the art (see, e.g., Physician's Desk Reference, 53rd ed., 1999).

In the first composition of matter, the agent can be "bound" to the 29p protein either covalently or non-covalently. Examples of covalent binding include, without limitation, N-terminal and/or C-terminal fusion proteins in the case of protein or peptide agents, and peptide or other chemical linkages in the case of agents that are nucleic acids or organic molecules. Methods of forming covalent bonds between proteins, proteins and

nucleic acid molecules, and proteins and organic molecules are routine in the art. In addition the agent can be bound to the 29p protein non-covalently. Examples of non-covalent bonds include, for example, those between 29p protein and an antibody directed thereto.

Delivery of the agent into a cell can be for any purpose, e.g., therapeutic, prophylactic, diagnostic, and labeling. As used herein, an entity is "delivered" into a eukaryotic cell if it traverses the cell membrane and enters the cytoplasm, nucleus, or other organelle thereof. Mechanisms of entry include, without limitation, cellular endocytosis.

The eukaryotic cell into which the instant composition is delivered can be any eukaryotic cell. In the preferred embodiment, the eukaryotic cell is a mammalian cell, e.g., a murine or human cell. Eukaryotic cells include, without limitation, Hela cells, fibroblasts, astrocytes, neurons, NB41 cells, and SupT-1 cells. Conditions under which the instant composition of matter will enter a eukaryotic cell include, for example, physiological conditions.

The present invention also provides a second composition of matter comprising a 29p protein having operably affixed thereto a lipid-soluble moiety which permits the protein to be anchored to a lipid membrane.

The present invention further provides a lipid vesicle comprising the second composition of matter anchored thereto via its lipid-soluble moiety, such that the 29p protein is situated on the vesicle's outer surface and

facilitates delivery of the vesicle's contents into a eukaryotic cell when the vesicle is contacted therewith. In the preferred embodiment, the vesicle's contents comprise an agent whose delivery into a cell is desired.

As used herein, the term "lipid-soluble moiety" shall mean an entity such as a hydrophobic polypeptide chain or a phospholipid capable of integrating within a lipid bilayer membrane. In one embodiment, the lipid-soluble moiety comprises a polypeptide chain bound to the N- or C-terminus of the 29p protein. Such fusion proteins can be made using known methods. The lipid-soluble moiety is "operably affixed" to the 29p protein if it does not interfere with the 29p protein's ability to enter a eukaryotic cell when contacted therewith. The second composition of matter is "anchored to a lipid membrane" in that it is immobilized with respect to the lipid membrane due to the lipid-soluble moiety's integration therein. Finally, as used herein, a vesicle's "contents" shall mean everything in or on the vesicle except the vesicle membrane and 29p protein anchored thereto.

The present invention also provides a monoclonal antibody which specifically binds to 29p protein. In one embodiment, the monoclonal antibody is labeled with a detectable marker. As used herein, the term "antibody" includes, without limitation, murine, human and humanized antibodies, and antigen-binding fragments thereof. Methods of generating monoclonal antibodies are well-known (30).

The present invention further provides a method for delivering an agent into a eukaryotic cell comprising

contacting the agent with the cell, wherein the agent has bound thereto 29p protein which enters the cell upon contact therewith, thereby delivering the agent into the cell.

The present invention further provides a method for causing a eukaryotic cell to secrete a desired protein in the form of a fusion protein, comprising introducing into the cell a vector for expressing a fusion protein that comprises the desired protein and 29p protein operably affixed thereto, whereby the cell expresses the fusion protein and the 29p protein thereof permits the fusion protein's exit from the cell, thereby causing the cell to secrete the desired protein in the form of a fusion protein. As used herein, the "secretion" of a protein by a cell shall mean the exit of that protein from the cell by any means. Expression vectors useful for carrying out the instant method are well known in the art (30).

As used herein, the term "fusion protein" shall mean a protein having a plurality of regions, each corresponding to a distinct protein or fragment thereof. Fusion proteins can include linker regions connecting the regions thereof, which are known in the art.

The present invention further provides a first pharmaceutical composition comprising (a) a composition of matter comprising 29p protein having bound thereto a therapeutic or prophylactic agent, which composition of matter enters a eukaryotic cell upon contact therewith, and (b) a pharmaceutically acceptable carrier.

The present invention further provides a second

pharmaceutical composition comprising a pharmaceutically acceptable carrier, and a lipid vesicle comprising (a) a therapeutic or prophylactic agent therein, and (b) a 29p protein having operably affixed thereto a lipid-soluble moiety, which protein (i) is anchored to the vesicle via its lipid-soluble moiety, (ii) is situated on the vesicle's outer surface, and (iii) facilitates delivery of the agent into a eukaryotic cell when the vesicle is contacted therewith.

The present invention further provides a method for treating a subject afflicted with a disorder comprising administering to the subject a therapeutically effective amount of the first or second pharmaceutical composition, wherein the therapeutic agent therein is known to ameliorate the disorder.

The present invention further provides a method for inhibiting the onset of a disorder in a subject comprising administering to the subject a prophylactically effective amount of the first or second pharmaceutical composition, wherein the prophylactic agent therein is known to inhibit the disorder's onset.

As used herein, "subject" shall mean any animal, such as a primate, mouse, rat, guinea pig, or rabbit. In the preferred embodiment, the subject is a human.

As used herein, "inhibiting the onset of a disorder" shall mean either lessening the likelihood of the disorder's onset, or preventing the onset of the disorder entirely. In the preferred emmbodiment, inhibiting the onset of a disorder means preventing its onset entirely.

As used herein, "treating" a disorder shall mean slowing, stopping, or reversing the disorder's progression. In the preferred embodiment, "treating" a disorder means reversing the disorder's progression, ideally to the point of eliminating the disorder itself. As used herein "ameliorating" and "treating" a disorder are equivalent.

Determining a therapeutically effective or prophylactically effective amount of the pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the therapeutically or prophylactically effective amount is an amount sufficient to deliver to the subject between about 1 μg/kg and 1 g/kg of the 29p protein therein. In another embodiment, the effective amount is an amount sufficient to deliver to the subject between about 100 μg/kg and 100 mg/kg of the 29p protein therein. In another embodiment, the effective amount is an amount sufficient to deliver to the subject between about 1 mg/kg and 10 mg/kg of the 29p protein therein. In another embodiment, the effective amount is an amount sufficient to deliver to the subject between about 10 mg/kg and 100 mg/kg of the 29p protein therein.

In the present invention, administering the instant pharmaceutical compositions can be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be performed, for example, intravenously, orally, via implant, transmucosally, transdermally, intramuscularly, and subcutaneously. The pharmaceutical carriers used in the instant pharmaceutical compositions are well known to those skilled in the art. The following drug delivery

systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition.

Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g. ethanol, propylene glycol and sucrose) and polymers (e.g. polycaprylactones, and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprylactone.

Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrilodone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

Transmucosal delivery systems include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Dermal delivery systems are preferred, and include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous

and nonaqueous solutions, lotions aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers, (e.g. fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N^{I},N^{II},N^{III}-tetramethyl-N,N^{I},N^{II},N^{III}-tetrapalmityl-spermine and dioleoyl phosphatidyl ethanolamine (DOPE)(GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethylammonium methylsulfate)(Boehringer Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL). Herein, the term "liposome" and "lipid vesicle" are used interchangebly. In the instant pharmaceutical compositions comprising 29p protein-containing lipid vesicles, the term "pharmaceutically acceptable carrier" refers to carriers other than liposomes.

Solutions, suspensions and powders for reconstitutable delivery systems include vehicles such as suspending agents (e.g., gums, zanthans, cellulosics and sugars), humectants (e.g. sorbitol), solubilizers (e.g., ethanol, water, PEG and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine), preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-

caking agents, coating agents, and chelating agents (e.g., EDTA).

The present invention further provides a nucleic acid molecule which hybridizes to at least a portion of a nucleic acid molecule encoding 29p protein.

In one embodiment, the nucleic acid molecule encoding 29p protein has the sequence shown in Figure 6. In a further embodiment, the nucleic acid molecule is complementary to the nucleic acid molecule having the sequence shown in Figure 6.

The invention further provides the nucleic acid molecule which hybridizes to at least a portion of a nucleic acid molecule encoding 29p protein, wherein the nucleic acid molecule is labeled with a detectable marker.

The present invention further provides a method for detecting the presence of a 29p protein-encoding nucleic acid molecule in a sample comprising the steps of (a) contacting the sample with the instant detectable nucleic acid molecule under conditions permitting it to hybridize to a 29p protein-encoding nucleic acid molecule if present in the sample, and (b) detecting the presence of any detectable nucleic acid molecule so hybridized, thereby detecting the presence of a 29p protein-encoding nucleic acid molecule in the sample.

Finally, the present invention provides a method for quantitatively determining the amount of 29p protein-encoding nucleic acid molecule in a sample comprising the steps of (a) contacting the sample with the instant

detectable nucleic acid molecule under conditions permitting it to hybridize to any 29p protein-encoding nucleic acid molecule present in the sample, (b) quantitatively determining the amount of detectable nucleic acid molecule so hybridized, and (c) comparing this amount to a known standard, thereby quantitatively determining the amount of 29p protein in the sample.

Conditions permitting nucleic acid hybridization are well known in the art and include, without limitation, physiological conditions (30). Detectable markers are known in the art, and include, without limitation, markers utilizing fluorescence and radiolabeling.

The known standard to which the amount of hybridized detectable nucleic acid molecule is compared can be, for example, one or more data points correlating known amounts of 29p protein-encoding nucleic acid molecule with the amounts of detectable nucleic acid molecule that hybridize therewith.

The definitions of terms set forth in the Detailed Description of the Invention are applicable wherever such terms occur herein, unless stated otherwise.

The present invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

The ORF29p protein is a 131 kdalton protein encoded by a 3612bp open reading frame in the varicella zoster virus (VZV). On the basis of nucleotide and amino acid homology the protein was predicted to be a homologue of ICP8, a well characterized single-stranded DNA-binding protein from herpes simplex virus (HSV). Subsequent biochemical studies have verified this to be the case. VZV and HSV are members of the family alphaherpesviridae.

We demonstrated that ORF29p was detected in peripheral nerves in the dermis in biopsy specimens taken from patients with chicken pox. Because of the role of this protein in the replication of virus DNA, which occurs in the nucleus of infected cells, this was an unexpected finding. When medium from cells infected with HSV or VZV was harvested, clarified by centrifugation and subsequently filtered to remove cells and large debris we were able to demonstrate that ORF29p, but not ICP8, was secreted from tissue culture cells infected in vitro, in vivo, and ex vivo. The clarified supernatants from cells infected with VZV but not HSV contained a protein with the mobility characteristic of ORF29p that reacted with antibody specific for this protein.

Subsequent assays of the filtered tissue culture medium from VZV infected cells demonstrated that the ORF29p protein present in the medium was able to enter cultivated human neurons as evidenced by immunohistochemical analysis. In similar, but unpublished, studies we have demonstrated that ORF29p is assimilated by human lymphocytes. In other unpublished

studies, we have shown that recombinant ORF29p, purified from insect cells infected with a baculovirus expressing the protein, is taken up by cultured human neurons and detected in both the cytoplasm and nucleus of these cells.

Thus, ORF29p has the potential to be used as a charon for the delivery of pharmaceuticals to a wide spectrum of human cells. For example, delivery of analgesics coupled to ORF29p to sensory nerves in areas of the body where there is intense local pain could provide local relief. These pharmaceuticals could include biological macromolecules and small molecules such as hormones, growth factors, enzymes, toxins, antiviral agents and chemical compounds to be used as drugs.

Example 1: Varicella-Zoster Virus Proteins in Skin Lesions: Implications for a Novel Role of ORF29p in Chickenpox

Skin biopsy samples from varicella-zoster virus (VZV)-infected patients examined by immunohistochemistry demonstrated VZV replication in nonepithelial cell types. ORF29p, a nonstructural nuclear protein, was found in nerves of two of six patients with chickenpox. In tissue culture, ORF29p was secreted by VZV-infected fibroblasts. Extracellular ORF29p can be taken up through endocytosis by human neurons, implying a novel role for this protein in pathogenesis.

To determine if, during primary infection, as in zoster, VZV infects endothelial cells and nerves in the dermis and to characterize the inflammatory cells in the

epidermis and dermis infected by VZV, we performed comparative immunohistochemical analyses of skin biopsy samples obtained from patients with chickenpox and zoster.

Comparative immunohistochemical analysis of chickenpox and zoster lesions. Six cases of chickenpox and eight cases of zoster were analyzed by immunohistochemistry using purified polyclonal antibodies generated against VZV proteins (18). Each specimen was analyzed for the presence of gC, a late gene product and component of the virus envelope (15); IE63p, a regulatory protein and component of the virus tegument (14); and ORF29p (13). In these specimens, antibodies to IE63p, ORF29p, and gC detected proteins in the expected intracellular compartments: ORF29p was found in cell nuclei (Fig. 1), gC was found in cell membranes and the cytoplasm, and IE63p was found in both the cell nucleus and cytoplasm (data not shown). VZV proteins were detected in epithelial cells, endothelial cells, nerves, and CD43+/CD68+ inflammatory cells in the epidermis and dermis in both chickenpox and zoster cases (Fig. 1 and 2). Among the six chickenpox specimens, all were positive for ORF29p, four were positive for IE63p, and five were positive for gC. Among the eight zoster specimens, all were positive for ORF29p, five were positive for IE63p, and five were positive for gC (Table 1).

TABLE 1. Detection of VZV proteins in skin biopsy samples[a]

Diagnosis	No.positive/no. examined	VZV protein[b]									
		IE63p			ORF29p				gC		
		EP	EN	NE	EP	EN	NE	WBC[c]	EP	EN	NE
CP	6/6	4	3	0	6	4	2	4	4	5	3
Z	8/8	5	1	0	8	4	0	5	5	5	4
G	0/5	0	0	0	0	0	0	0	0	0	0
HSV-2	0/3	0	0	0	0	0	0	0	0	0	0

[a]Tissues from patients with clinical and histopathological diagnoses of chickenpox (CP), zoster (Z), Grover's disease (G) or HSV-2 were subjected to immunohistochemical analysis for immediate-early (IE63p), early (ORF29p), and late (gC) virus proteins in epithelial (EP), endothelial (EN), or inflammatory cells expressing CD43 (WBC) or in dermal nerves (NE). [b]Results are expressed as the absolute number of biopsy samples with detectable protein. Zero indicates the absence of detectable protein. [c]VZV-infected WBC from two chickenpox cases and two zoster cases were found to express CD68 and not CD3 or CD20.

These results are consistent with our experience concerning the affinities of these antibodies for their target proteins. Five cases of Grover's disease, a noninfectious dermatosis, and three cases of HSV infection were included as controls and were negative (Table 1), confirming that each antibody signal was specific. Detection of ORF29p, a DNA-binding protein not present in the virion (13), in the nuclei of infected cells demonstrates that VZV replication occurs in endothelial cells, epithelial cells, and cells of the monocyte/macrophage lineage expressing surface CD43 and

CD68. Although VZV genomes were detected in circulating lymphocytes of patients with chickenpox and zoster (16, 20) we detected VZV proteins only in CD43+/CD68+ cells and not in cells expressing CD3 or CD20 in these specimens.

No differences in immunohistochemical staining were appreciated between specimens of chickenpox and zoster except for the presence of ORF29p in peripheral nerves in the dermis in two of the chickenpox specimens. ORF29p was detected in the Schwann cells and axons of nerves in these two cases (Fig. 3A), which included a biopsy sample that was obtained 2 days following the onset of the rash. Cytoplasmic localization of ORF29p is not consistent with the presence of replicating VZV or of formed virions. In contrast, ORF29p was not detected in axons or Schwann cells in the seven zoster biopsy samples with peripheral nerves apparent in the analyzed sections (Fig. 3C). The absence of ORF29p from axons during zoster was not surprising, as this protein localizes to the nucleus of productively infected cells, including neurons containing reactivating virus (18), and is not detected in virions (13). As expected, gC was found in both axons and Schwann cells of nerves in three chickenpox cases and four zoster cases (Fig. 3B and D). Detection of gC indicates the presence of virions or replicating VZV at these sites. The significance of infection of Schwann cells in chickenpox and zoster is unclear. Schwann cells cultivated in tissue culture are permissive for VZV infection (4), but the role of these cells in pathogenesis is not known.

Secretion of ORF29p by infected cells in tissue culture. Clinical and laboratory evidence suggests that DRG are

infected via the peripheral nerves during the exanthem of chickenpox (11, 21, 26). Although hematogenous and axonal spread of virus are not mutually exclusive, given the small numbers of VZV-infected circulating PBMCs (16, 20), it seems unlikely that a substantial number of neurons are infected without amplification in the epidermis and dermis. Nonetheless, the exact mechanism by which VZV reaches DRG remains unsettled.

Entry of virus particles into peripheral axons during chickenpox cannot account for the presence of ORF29p at this site because this protein is not a component of the virion (13). Assuming that the virus spreads from the skin to the peripheral nerves during the exanthem, the appearance of ORF29p in the nerve within 2 days of rash onset is surprising because of the distance between the peripheral axon and the sensory neuron in the DRG. By analogy with HSV, it is thought that VZV entering the axon in the epidermis travels by retrograde axonal transport (2) at a rate of 200 to 400 mm/day (28). Additional time would be required for VZV proteins to be produced in the neuron in the DRG and then to travel to the dermis and epidermis by anterograde axonal transport. If the DRG were infected during viremia prior to the onset of the rash, virus replication in the neuron and anterograde axonal transport of VZV proteins could occur. However, this would not explain the presence of ORF29p in peripheral axons, because ORF29p localizes to the nucleus rather than to the cytoplasm during productive infection (18). Moreover, ORF29p was not found in peripheral axons during zoster.

We therefore postulated that ORF29p may be secreted by

VZV-infected cells in the dermis or epidermis and enter peripheral axons by endocytosis. In order to test whether ORF29p was secreted by VZV-infected cells, tissue culture media from uninfected human embryonic lung fibroblasts (HELF) or HELF infected with VZV or HSV-1 was clarified by centrifugation and filtration to remove detached cells. Immunoprecipitation with antibodies to ORF29p, ORF21p (a putative VZV accessory DNA binding protein), or ICP8 (the HSV-1 homologue of ORF29p) was performed and the precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. ORF29p was detected in culture supernatants of infected cells but not in culture supernatants of uninfected cells (Fig. 4). ORF21p was not detected in supernatants of VZV-infected cells, and ICP8 was not detected in supernatants of HSV-1-infected cells. Thus, ORF29p is secreted by infected fibroblasts in tissue culture.

Endocytosis of secreted ORF29p by neurons in tissue culture. Filtered tissue culture medium from VZV-infected HELF and LysoTracker Red DND-99 (Molecular Probes, Eugene, Oreg.), a label for acidic endocytic vesicles, were applied to cultivated human neurons (hNTs) (Stratagene, La Jolla, Calif.) to determine if secreted ORF29p entered neurons by endocytosis. After incubating the hNTs with the filtered culture medium and LysoTracker for 2 h, the cells were examined by immunohistochemistry for the presence of ORF29p. ORF29p was detected in cytoplasmic vesicles (Fig. 5A and D) that colocalized with LysoTracker (Fig. 5C). ORF29p was not found in untreated hNTs (Fig. 5E). Therefore, extracellular ORF29p can enter hNTs by endocytosis, supporting our hypothesis that the presence

of this protein in peripheral axons may result from its assimilation from surrounding cells that are infected with VZV.

Our results illustrate key steps of VZV pathogenesis. During chickenpox, VZV infects epithelial cells, endothelial cells, cells of the monocyte/macrophage lineage, and nerves of the skin. After infecting the neuron, the virus enters latency. In some individuals, the virus reactivates in one or more neurons, travels via the axon to the skin, and infects the epithelial cells.

In addition, endothelial cells are infected in zoster, which could potentially spread virus to other areas. That VZV does not typically spread outside of the dermatome during zoster implies that host immunity effectively halts cell-to-cell spread. This study suggests that entry of VZV into the nervous system during primary infection may not rely solely on axonal transport of mature virions from the skin during chickenpox, because ORF29p was present in axons early in the course of the rash.

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